AVIAN DISEASES 54:527–531, 2010

Research Note—

Truncation of the NS1 Protein Converts a Low Pathogenic Avian Influenza Virus into a Strong Interferon Inducer in Duck Cells

Sébastien Mathieu Soubies, Christelle Volmer, Jean-Luc Guérin, and Romain Volmer[A]

INRA, UMR 1225, Interactions Hôtes Agents Pathogènes, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, 31076 Toulouse Cedex, France

Received 23 March 2009; Accepted and published ahead of print 5 July 2009

SUMMARY. The NS1 protein of influenza A viruses is known as a nonessential virulence factor inhibiting type I interferon (IFN) production in mammals and in chicken cells. Whether NS1 inhibits the induction of type I IFNs in duck cells is currently unknown. In order to investigate this issue, we used reverse genetics to generate a virus expressing a truncated NS1 protein. Using the low pathogenic avian influenza virus A/turkey/Italy/977/1999 (H7N1) as a backbone, we were able to rescue a virus expressing a truncated NS1 protein of 99 amino acids in length. The truncated virus replicated poorly in duck embryonic fibroblasts, but reached high titers in the mammalian IFN-deficient Vero cell line. Using a gene reporter system to measure duck type I IFN production, we showed that the truncated virus is a potent inducer of type I IFN in cell culture. These results show that the NS1 protein functions to prevent the induction of IFN in duck cells and underline the need for a functional NS1 protein in order for the virus to express its full virulence.

RESUMEN. *Nota de Investigación*—El truncamiento de la proteína NS1 convierte a un virus de la influenza aviar de baja patogenicidad en un potente inductor de interferón en células de pato.

La proteína NS1 de virus de la influenza A es reconocida como un factor de virulencia no esencial que inhibe la producción de interferón tipo I en células de mamíferos y de pollos. Actualmente, se desconoce si la proteína NS1 inhibe la inducción de interferón tipo I en células de pato. Para investigar esta interrogante, se utilizó la genética reversa para generar un virus que expresaba la proteína NS1 truncada. Mediante la utilización del virus de la influenza aviar de baja patogenicidad A/Pavo/Italia/977/1999 (H7N1) como base, se pudo rescatar un virus que expresaba la proteína NS1 truncada que tenía una extensión de 99 aminoácidos. El virus truncado se replicó de manera inadecuada en fibroblastos de embriones de pato, pero alcanzó títulos altos en la línea celular de células de mamífero Vero que era deficiente en interferón. Utilizando un sistema indicador de genes para medir la producción de interferón tipo I de pato, se demostró que el virus truncado es un potente inductor de interferón en los cultivos celulares. Estos resultados muestran que la proteína NS1 funciona previniendo la inducción de interferón en células de pato y subraya la necesidad de una proteína NS1 funcional para que el virus exprese su virulencia completa.

Key words: influenza, avian, reverse genetics, NS1, duck, interferon

Abbreviations: aa = amino acid(s); DEF = duck embryonic fibroblasts; DMEM = Dulbecco's modified Eagle's medium; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HEK = human embryonic kidney; hpi = hours post-infection; IFN = interferon; IU = international unit; LPAI = low pathogenic influenza virus; m.o.i. = multiplicity of infection; NEP = nuclear export protein; n.i. = not infected; NP = nucleoprotein; NS = non-structural; NS1 = non-structural protein 1; p.f.u. = plaque forming unit; RT-PCR = reverse transcription–polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPCK = L-1-tosylamido-2-phenylethyl chloromethyl ketone; wt = wild-type

Influenza viruses are the causative agents of epizootics and a potential source of zoonosis. Although the recent outbreaks of highly pathogenic H5N1 virus have created a great deal of interest about the virulence factors in mammals, little is known about the interaction between influenza viruses and their natural reservoir, namely waterfowl (12). In particular, the extent of the innate immune response and its consequences on the kinetics of infection in ducks are unknown.

In mammals, influenza nonstructural protein 1 (NS1) is described as a nonessential virulence factor, able to inhibit the innate antiviral immune response of infected cells. This is mainly achieved through the interaction of the NS1 effector domain with several cellular proteins. This effector domain has been mapped to amino acids 73 to 230, which correspond to the C-terminal region of the protein (6). In ducks, the interaction between NS1 and the innate immune response remains to be evaluated.

[A]Corresponding author: r.volmer@envt.fr

Using reverse genetics, NS1-truncated viruses have been generated and their phenotype studied in several species including mouse (5), horse (13), pig (17), and macaque (1). These viruses proved to be attenuated and were strong interferon (IFN) inducers, underlining the role of NS1 as an antagonist of the innate immune response. NS1 also acts as a virulence factor inhibiting IFN production in chicken cells, as evidenced by studies using a natural low pathogenic influenza virus (LPAI) with a truncated NS1 protein, or an H7N7 influenza virus with a truncated NS1 generated by reverse genetics (3,10,11). At present, the consequences of NS1 truncation on infection of ducks remain unknown.

To answer this question, we used the LPAI virus A/turkey/Italy/977/1999(H7N1) as a backbone to generate, by reverse genetics, an LPAI virus expressing a truncated NS1 protein, of 99 amino acids (aa) in length, named H7N1 1–99. Compared with the wild-type (wt) virus, the mutant replicated poorly in primary duck embryonic fibroblasts (DEF), but reached high titers in the IFN-deficient Vero cell line. The mutant virus appeared as a very potent type I IFN

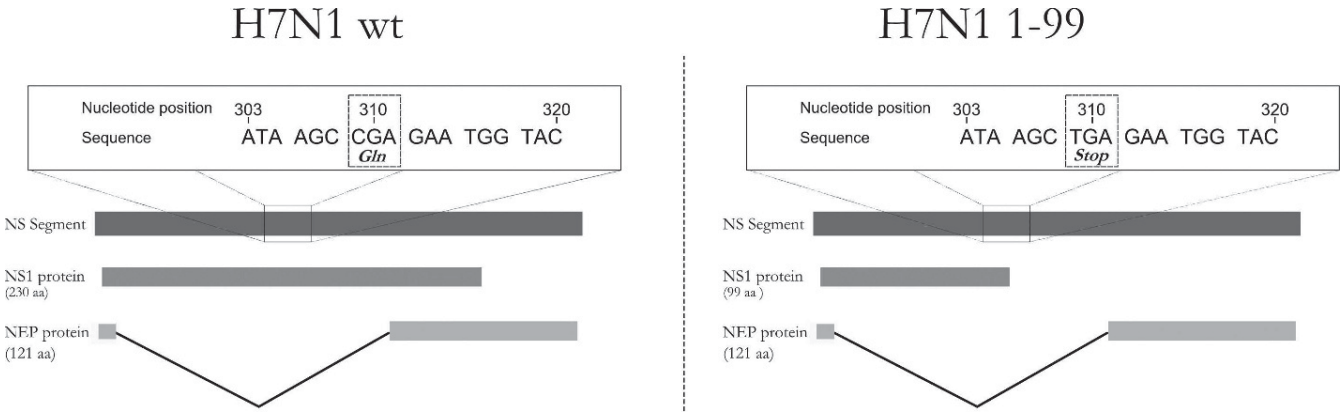

Fig. 1. Production of a mutated virus expressing a 99-aa truncated NS1 Protein. Schematic of H7N1 wt and H7N1 1–99 NS segment. Influenza A NS segment encodes two proteins, NS1 and NEP, which are represented. NEP is obtained by the splicing of NS1 mRNA. Wild-type NS1 is 230 aa long. The mutation performed on the NS segment occurs within the intronic region of the NEP open reading frame and, therefore, only affects NS1, resulting in a truncation after position 99.

inducer compared with the wt virus when infecting duck cells. This study illustrates the importance of the NS1 protein as an antagonist of the host innate immune response in ducks.

MATERIALS AND METHODS

Cells, viruses, and antibodies. DEF were obtained from 10-day-old Pekin duck (*Anas platyrhynchos domestica*) embryos. All cells, including human embryonic kidney (HEK) 293T (American Type Culture Collection, Manassas, VA) and Vero cells (American Type Culture Collection) were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with penicillin (10^4 U/ml), streptomycin (10 mg/ml), and 10% fetal bovine serum at 37 C. The following antibodies were used: polyclonal rabbit serum directed against NS1 (kindly provided by Dr. D. Marc [INRA, Tours, France]), polyclonal rabbit anti-nucleoprotein (NP; kindly provided by Dr. G. Whittaker [Cornell University, Ithaca, NY]), mouse monoclonal antibody directed against NP (Argene, Verniolle, France), and mouse monoclonal antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Millipore, Billerica, MA). The LPAI virus A/turkey/Italy/977/1999 (H7N1) was a kind gift of Dr. I. Capua (Istituto Zooprofilattico Sperimentale Delle Venezie, Legnaro, Italy).

Viruses and reverse genetics. Viral genome was extracted from the allantoic fluid of chicken embryonated eggs infected with the LPAI A/turkey/Italy/977/1999 (H7N1) virus (third egg passage) using the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The eight viral segments were subsequently reverse-transcribed using the Superscript II enzyme (Invitrogen, Carlsbad, CA) and Uni 12 as a universal primer for viral genome segments (8). Using specific primers for each segment described by Hofmann *et al.* (8), the viral genome was amplified with the Phusion high fidelity polymerase (Finnzymes, Espoo, Finland). Each segment was then cloned into a shuttle plasmid (Strataclone blunt PCR cloning kit, Stratagene). After sequencing, plasmids containing segments identical to the published sequences of A/turkey/Italy/977/1999 (H7N1) were used to clone each of the viral segments into the pHW2000 vector, kindly provided by Prof. Webster (St. Jude Children Research Hospital, Memphis, TN) (7). In order to generate a mutant virus, site-directed mutagenesis was performed on the NS segment using the QuickChange II kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. The introduced mutation is presented in Fig. 1. The plasmid was then sequenced to ensure the absence of unwanted mutations.

Rescue of infectious viruses. HEK 293T cells were transfected with 0.3 μg of each of the eight plasmids corresponding to the viral segments, using the lipophilic transfection reagent LTX (Invitrogen) with Plus reagent (Invitrogen), as described in the manufacturer's protocol. To avoid multicycle replication in HEK 293T, no trypsin was added to the culture medium at this step. Three wells were transfected for each rescue and were respectively scraped at 24, 36, and 48 hours posttransfection. Scraped cells and culture medium were then transferred on DEF grown in OptiMEM supplemented with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin (2 μg/ml; Thermo Scientific, Rockford, IL) to increase viral titer. In order to produce viral stock, H7N1 wt was further inoculated to embryonated eggs, whereas H7N1 1–99 was used to infect Vero cells. Viral titers were measured by plaque assay on Vero cells. We verified the identity of amplified viruses by sequencing amplicons of each viral gene segment using reverse transcription–polymerase chain reaction (RT-PCR).

Infections. All infections were performed in DMEM supplemented with 0.2% bovine serum albumin, and TPCK trypsin (2 μg/ml) was added in the case of multicycle growth analysis.

Hemagglutination assay. Cell culture supernatants were harvested, serially diluted in phosphate-buffered saline, and assayed for hemagglutination of 1% horse red blood cells in V-bottom, 96-well plates by adapting a described protocol (9).

Western blotting. DEF infected, or not (n.i.), with either H7N1 wt or H7N1 1–99, were lysed 4, 8, and 20 hours postinfection (hpi) in Ipegal buffer in the presence of protease inhibitors (Roche Diagnostics, Meylan, France). Cell lysates were treated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting. Polyvinylidene fluoride membranes were incubated with rabbit anti-NS1 polyclonal serum (1/10,000), a rabbit anti-NP polyclonal serum (1/5,000), and mouse anti-GAPDH (1/20,000). Horseradish peroxidase-conjugated relevant antibodies were added and revelation was done using the Supersignal West Dura kit (Thermo Scientific).

Titration of type I IFNs by reporter assay. Recombinant duck IFN-α was produced by transfecting (monkey) COS cells with a plasmid encoding IFN-α, kindly provided by Dr. U. Schultz (Universität Freiburg, Germany) (15). Supernatant was harvested and titration done on DEF by measuring protection against vesicular stomatitis virus-induced cytopathic effect. The titer reached 12,000 IU/ml. To determine the amount of type I IFNs produced in the supernatant of infected cells, a reporter assay was used. DEF were cotransfected by electroporation (BioRad GenePulser X cell; Biorad, Marnee-la-Coquette, France) with a plasmid containing the sequence of firefly luciferase under the control of chicken Mx promoter, MxLuc, kindly provided by P. Staeheli (Universität Freiburg, Germany), and a plasmid containing the sequence of *Renilla* luciferase under the control of herpes simplex virus TK promoter (Promega) (16). Sixteen hours after transfection, cells were stimulated with supernatant from cells infected with an m.o.i. of 2 (virus in the supernatant was inactivated by acid treatment at pH = 2), or recombinant duck IFN-α, for 16 hr. The activity of firefly luciferase was then measured on a Tecan Infinite

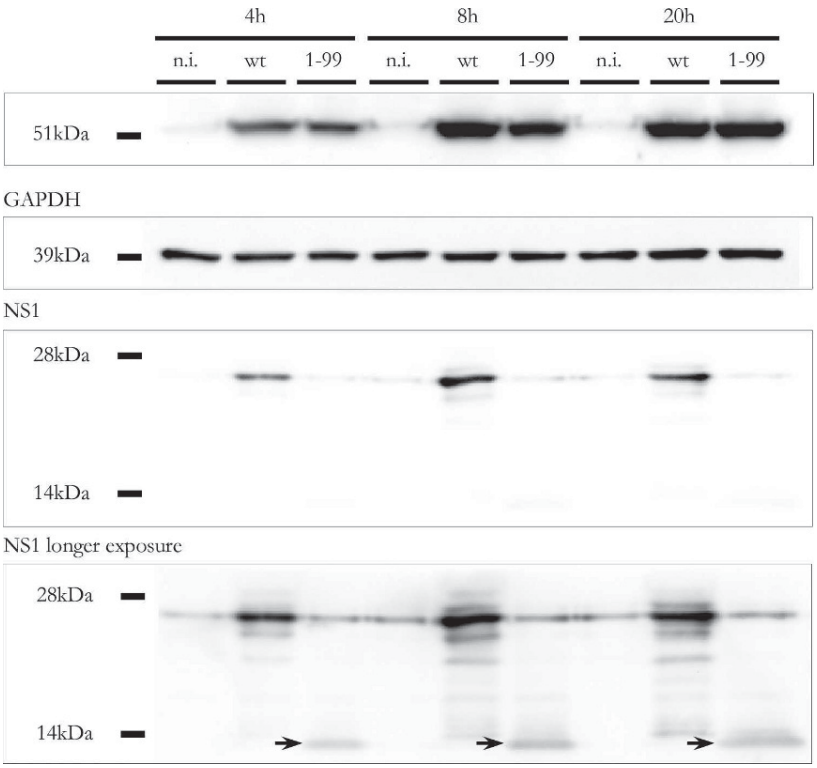

Fig. 2. Replication of H7N1 1–99 *in vitro*. Western blots for NP, GAPDH, and NS1: DEF were infected or not (n.i.) with wt or mutant virus and lysed respectively at 4, 8, and 20 hpi. Cell lysates were analyzed by SDS-PAGE, followed by Western blot analysis for NP (upper), GAPDH (middle), and NS1 (lower panel). In the case of NS1, a longer exposure time (lower panel) allowed us to visualize a band corresponding to the truncated protein (arrow).

200 96 plate reader (Tecan, Männedorf, Switzerland) using the Dual-Glo Luciferase assay system (Promega, Madison, WI) and was normalized by the *Renilla* activity.

RESULTS

Production of a mutant virus expressing a truncated NS1 protein by reverse genetics. We mutated the NS segment in order to produce a 99-aa truncated NS1 protein, following a strategy successfully used by others (10,13,17,18). The mutation occurs in the intronic region of the nuclear export protein (NEP) so that the modification alters only NS1. Deleting this large portion of the protein allows the conservation of some functions of the NS1 protein carried on its N-terminal region, such as double-stranded RNA binding (Fig. 1) (6). Plasmid-based rescue was performed on the highly transfectable HEK 293T cell line to allow vector expression: Infectious virus produced by transfected cells was then amplified on DEF in the presence of trypsin to allow multicycle growth. DEF supernatant reached a titer of 2.5×10^5 plaque-forming units (p.f.u.) per ml in the case of H7N1 wt, whereas H7N1 1–99 replicated poorly, with a titer of 10^3 p.f.u./ml. The reduced titer of H7N1 1–99 could be due to the IFN response of DEF. Therefore, we decided to further amplify the mutant virus on the IFN-deficient cell line Vero. H7N1wt was amplified on embryonated eggs. By these means, H7N1 wt reached 3.3×10^7 p.f.u./ml and H7N1 1–99 titrated at 10^7 p.f.u./ml.

We checked the viability of the mutant virus by infecting DEF at an m.o.i. of two. The expression of NP and NS1 proteins was then analyzed by Western blot (Fig. 2). The level of NP expression appeared similar for both viruses at 4 hpi, 8 hpi, and 20 hpi. NS1 protein was detected specifically in H7N1 wt infected cells as a band migrating just below the 28 kDa marker, corresponding to full-length NS1. A longer exposure time resulted in background signal, present in both infected and control cells. However, a weak band appeared at approximately 13 kDa, specifically in the case of H7N1 1–99 infected cell lysate (Fig. 2, arrow); this band most likely corresponded to NS1, as its size matched the theoretical molecular weight of the truncated protein. These data illustrate the effectiveness of the mutation carried out on the viral genome. Collectively, these results show that the mutant virus expressing a truncated NS1 protein is able to infect DEF and to replicate in these cells.

H7N1 1–99 replicates poorly in DEF but reaches high titers in Vero cells. To investigate the replicative capacity of the mutant virus, infections were performed in DEF and Vero cells at an m.o.i. of 0.01, and HA titer of the supernatant was measured at different time-points (Fig. 3). In DEF, the wt virus reached an HA titer of 64 within 24 hr and maintained this titer until the end of the experiment. In contrast, the titer of the mutant virus peaked at 32 HA units and then decreased to 16. In Vero cells, both viruses reached a high titer (1024 HA units) by 72 hr. These data show, in agreement with the findings from other groups, that the replicative capacity of the mutant virus is altered in IFN-competent cells such as DEF, but is similar to wt virus in the IFN-deficient Vero cell line (5,10).

Truncation of the NS1 protein converts LPAI into a strong IFN inducer in DEF. In previous works, several groups have shown that viruses expressing a truncated NS1 elicited a major type I IFN response in infected cells compared to their wild-type counterparts (1,5,10,11,13,17). In order to check for the consequences of our

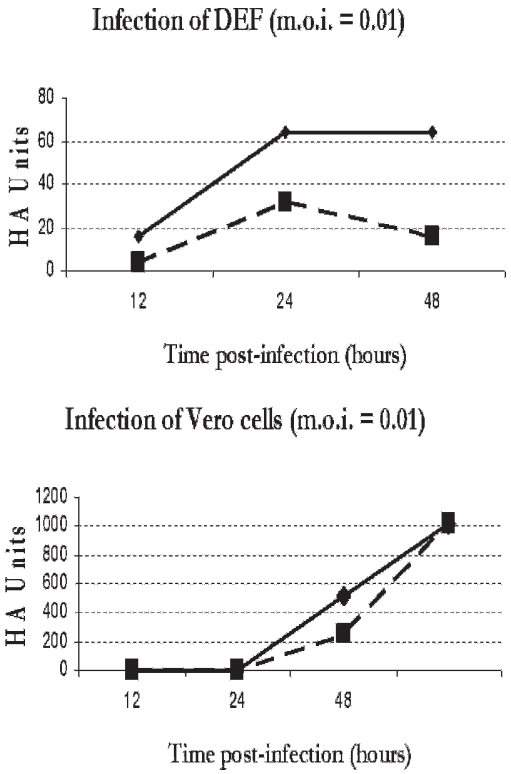

Fig. 3. Infection and multicycle replication of H7N1 wt (plain line) and H7N1 1–99 (dashed line) viruses. DEF and Vero were infected at an m.o.i. of 0.01 by either virus. Supernatants were collected at several time-points and analyzed for their HA titer.

mutation in duck cells, we used a reporter assay to measure type I IFN production of infected DEF. Stimulating DEF with recombinant duck IFN-α resulted in a detectable signal for a 5 IU/ml concentration (Fig. 4). The response appeared linear up to a concentration of 500 IU/ml. Supernatant from wt virus infected cells harvested 20 hpi contained a concentration of type I IFNs of about 50 IU/ml. In contrast, supernatant from mutant virus-infected cells resulted in a very strong response in our assay, corresponding to a concentration much higher than 500 IU/ml. These data show that truncating the NS1 protein converts an LPAI virus into a strong type I IFNs inducer in duck cells.

DISCUSSION

By using reverse genetics, we were able to create a mutant avian influenza virus expressing a truncated NS1 protein. The mutant virus displayed an impaired replication in DEF and induced high titers of type I IFNs. This shows that NS1 inhibits IFN production in duck cells, as in chicken and mammalian cells. The reduced growth of the H7N1 1–99 virus during multi-cycle analyses in DEF is most likely due to the synthesis of IFN-induced antiviral effectors. At present, the nature of these effectors in duck cells remains unknown. Surprisingly, Mx protein, which is a potent IFN-induced antiviral protein in mammals, appears to be inactive in ducks (2).

Previous studies have shown that LPAI viruses with a truncated NS1 were attenuated in chickens (3,20). Although LPAI are nonpathogenic in ducks, it would be interesting to evaluate the impact of NS1 truncation on the tissue tropism and on the duration of infection (12). In addition, the H7N1 1–99 is a strong IFN inducer and, thus, represents an interesting means to study the innate immune

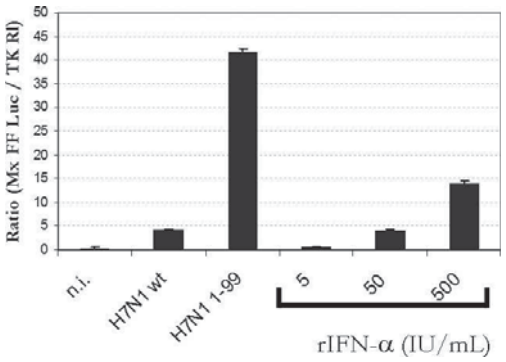

Fig. 4. NS1 truncation turns H7N1 wt into a strong IFN inducer. Quantification of IFN titers: Mx firefly-luciferase activity normalized by *Renilla* luciferase activity was measured in cells stimulated with supernatant from infected DEF (left part), or with increasing concentrations of rIFN-α (right part). Data represent the mean of triplicates; error bars represent standard deviation.

response during influenza virus infection of ducks, a phenomenon poorly described in this species. A particularly efficient innate immune response may explain why ducks survive infections with highly pathogenic influenza viruses that are lethal to other avian species (19). Finally, viruses expressing a truncated NS1 proved to be both attenuated and immunogenic when administered to mammals and chickens and, therefore, are promising attenuated vaccine strains (1,4,14,18,20). The potential of the H7N1 1–99 virus as a vaccine strain could be the subject of further studies in ducks and other avian species.

REFERENCES

1. Baskin, C. R., H. Bielefeldt-Ohmann, A. Garcia-Sastre, T. M. Tumpey, N. Van Hoeven, V. S. Carter, M. J. Thomas, S. Proll, A. Solorzano, R. Billharz, J. L. Fornek, S. Thomas, C. H. Chen, E. A. Clark, K. Murali-Krishna, and M. G. Katze. Functional genomic and serological analysis of the protective immune response resulting from vaccination of macaques with an NS1-truncated influenza virus. J. Virol. 81:11817–11827. 2007.

2. Bazzigher, L., A. Schwarz, and P. Staeheli. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. Virology 195:100–112. 1993.

3. Cauthen, A. N., D. E. Swayne, M. J. Sekellick, P. I. Marcus, and D. L. Suarez. Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J. Virol. 81:1838–1847. 2007.

4. Chambers, T. M., M. Quinlivan, T. Sturgill, A. Cullinane, D. W. Horohov, D. Zamarin, S. Arkins, A. Garcia-Sastre, and P. Palese. Influenza A viruses with truncated NS1 as modified live virus vaccines: pilot studies of safety and efficacy in horses. Equine Vet. J. 41:87–92. 2009.

5. Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252:324–330. 1998.

6. Hale, B. G., R. E. Randall, J. Ortin, and D. Jackson. The multifunctional NS1 protein of influenza A viruses. J. Gen. Virol. 89:2359–2376. 2008.

7. Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. 97:6108–6113. 2000.

8. Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146:2275–2289. 2001.

9. Killian, M. L. Hemagglutination assay for the avian influenza virus. Methods Mol. Biol. 436:47–52. 2008.

10. Kochs, G., I. Koerner, L. Thiel, S. Kothlow, B. Kaspers, N. Ruggli, A. Summerfield, J. Pavlovic, J. Stech, and P. Staeheli. Properties of H7N7

influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. J. Gen. Virol. 88:1403–1409. 2007.

11. Marcus, P. I., J. M. Rojek, and M. J. Sekellick. Interferon induction and/or production and its suppression by influenza A viruses. J. Virol. 79:2880–2890. 2005.

12. Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. Osterhaus, and R. A. Fouchier. Global patterns of influenza A virus in wild birds. Science 312:384–388. 2006.

13. Quinlivan, M., D. Zamarin, A. Garcia-Sastre, A. Cullinane, T. Chambers, and P. Palese. Attenuation of equine influenza viruses through truncations of the NS1 protein. J. Virol. 79:8431–8439. 2005.

14. Richt, J. A., P. Lekcharoensuk, K. M. Lager, A. L. Vincent, C. M. Loiacono, B. H. Janke, W. H. Wu, K. J. Yoon, R. J. Webby, A. Solorzano, and A. Garcia-Sastre. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J. Virol. 80: 11009–11018. 2006.

15. Schultz, U., J. Summers, P. Staeheli, and F. V. Chisari. Elimination of duck hepatitis B virus RNA-containing capsids in duck interferon-alpha-treated hepatocytes. J. Virol. 73:5459–5465. 1999.

16. Schumacher, B., and P. Staeheli. Domains mediating intramolecular folding and oligomerization of MxA GTPase. J. Biol. Chem. 273: 28365–28370. 1998.

17. Solorzano, A., R. J. Webby, K. M. Lager, B. H. Janke, A. Garcia-Sastre, and J. A. Richt. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J. Virol. 79:7535–7543. 2005.

18. Steel, J., A. C. Lowen, L. Pena, M. Angel, A. Solorzano, R. Albrecht, D. R. Perez, A. Garcia-Sastre, and P. Palese. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J. Virol. 83:1742–1753. 2009.

19. Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J. Virol. 79:11269–11279. 2005.

20. Wang, L., D. L. Suarez, M. Pantin-Jackwood, M. Mibayashi, A. Garcia-Sastre, Y. M. Saif, and C. W. Lee. Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine in poultry. Vaccine 26:3580–3586. 2008.

ACKNOWLEDGMENTS

We thank D. Marc, G. Whittaker, U. Schultz, P. Staeheli, I. Capua, and W. Dundon for the generous gift of reagents; and S. Bertagnoli and C. Camus for critical reading of the manuscript.